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14. ABSTRACT We have proposed to developed indicator cell lines that would allow for the high throughput screening (HTS) for compounds that potentially inhibit prostate cancer (CaP) metastasis. The cell lines are based on stably expressing a construct containing the promoter of SSeCKS/ gravin/AKAP12- a metastasis-suppressor gene downregulated in CaP progression- linked to a green fluorescence protein (GFP), plus a control reporter, in metastatic CaP cells, and then screening for compounds that induce GFP. We also proposed to characterize the pathways controlling SSeCKS expression in CaP progression. UPDATE: Our data indicate that SSeCKS re-expression can be induced in CaP cell lines using inhibitors of histone deacetylation (TSA) but not by inhibitors of methylation (5-aza-C). We have now produced stable indicator C4-2 and DU145 cells, the latter of which is more inducible by TSA. We have also characterized the cis- and trans-acting elements of the human SSeCKS promoter required for transcriptional suppression in CaP cells.						
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Introduction

We have proposed to construct indicator prostate cancer (CaP) cell lines that could be used to identify novel drugs that could inhibit parameters of oncogenic and metastatic growth. The cell lines are based on the stable expression of the promoter from the SSeCKS/gravin/AKAP12 metastasis-suppressor fused to a green fluorescent protein (GFP) reporter, plus a control reporter plasmid. In a high throughput screen (HTS), compounds that induce GFP expression but have no major effect on the control reporter would be identified for further analysis as potential inhibitors of CaP progression. A second major aim of our study is to use the SSeCKS/gravin/AKAP12 α promoter to characterize the signaling pathways as well as the *cis*- and *trans*-acting mechanisms leading to transcriptional downregulation in CaP cells. This analysis included a determination whether CaP-specific gene silencing involves hypermethylation of CpG islands in the SSeCKS promoters or changes in chromatin acetylation.

Body

I will first describe the progression we have made to produce and test the CaP indicator lines. Our first task was to produce CaP lines stably expressing the human SSeCKS/gravin α promoter-GFP, plus a control reporter (described in Task 1 in the Statement of Work). The promoter was cloned into the pEGFP vector and in transient expression assays, this construct was shown to express high levels of green fluorescence in untransformed cells (murine NIH3T3 and human P69SV40T prostate epithelial) yet low GFP levels in LNCaP and C4-2 CaP cells. Instead of the originally intended control reporter (SEAP), we chose to use an RFP (red fluorescent protein) reporter (Clontech) which we fused to the TK promoter. In transient assays, almost equal RFP expression levels were detected in all untransformed and CaP cell lines (data not shown). We then stably transfected P69, LNCaP and C4-2 cells with both plasmids, selected for neo^R colonies, and then FACS sorted pooled colonies for the desired phenotype: C4-2 and LNCaP cells were selected for low GFP, high RFP; P69 cell were selected for high GFP and RFP. 5 individual clones from each were expanded.

We then tested a panel of signal transduction inhibitors, differentiating agents and transcriptional deregulators for their ability to re-induced GFP and endogenous SSeCKS expression in the CaP cells while having minimal effect on RFP expression in either the CaP or P69 cells. Prior to this analysis, we first developed probes to detect changes in the levels of endogenous human SSeCKS α and β mRNA and protein isoforms. These included isoform-specific PCR primer sets (see Fig. 3), and from previous studies, polyclonal antibodies (Ab) that detected both α and β protein isoforms (3) as well as an Ab that detected only the α isoform (2).

Although data from other groups had shown cases where SSeCKS expression was suppressed by either promoter hypermethylation (1) or histone deacetylation (4), our data indicated that treatment of CaP cells with methylation inhibitors (5-aza-C or deoxy-5-aza-C) had no effect on transcript levels where the histone deacetylase inhibitor, TSA, derepressed SSeCKS transcript levels roughly 6- to 10-fold (although, still significantly lower than untreated P69 cell levels). We then tested a panel of pathway inhibitory drugs to help define which pathways are responsible for SSeCKS downregulation in CaP and v-Src cells. Unfortunately,

endogenous SSeCKS or SSeCKS/GFP was not induced by inhibitors of Src, MEK, JNK, PI3K, or by differentiating agents (at-retinoic acid, calcitriol) (data not shown). Thus, we had to conclude that LNCaP and C4-2 cells were not ideal since the traditional oncogenic signaling mechanisms controlling gene transcription were not targetable. One possible explanation is that LNCaP and C4-2 cells have severely downregulated SSeCKS/gravin mRNA and protein levels, approaching a 100-fold decrease, possibly attributable to the loss of 6q21-ter (where SSeCKS maps) in one of two alleles.

An alternative, which we suggested in our proposal, was to use other metastatic CaP lines, such as DU145, which expresses downregulated levels of SSeCKS, although more like 8-fold

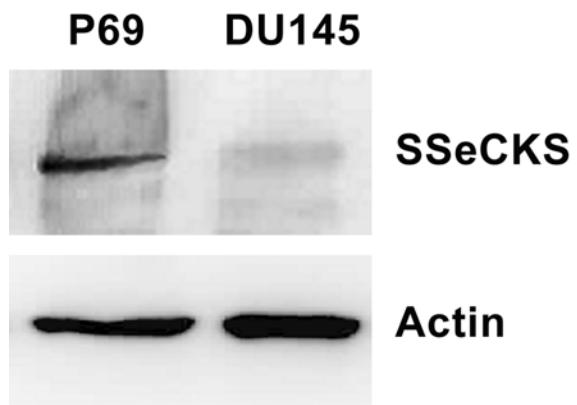


Figure 1. Immunoblot of lysates from untransformed P69 human epithelial versus DU145 CaP cells probed for either SSeCKS or actin (as a loading control).

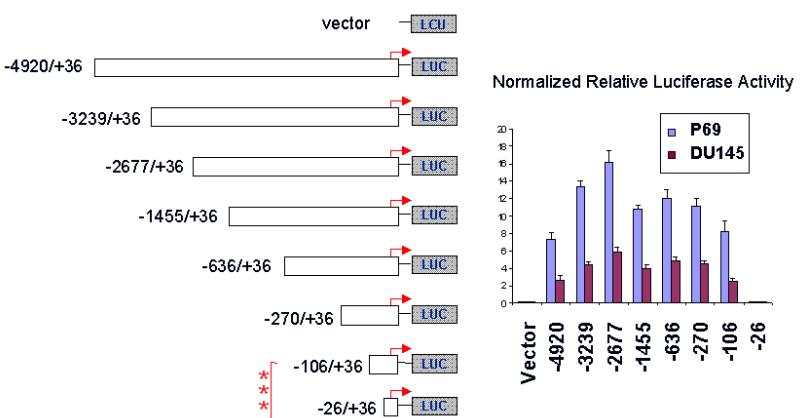


Figure 2. Control of SSeCKS transcriptional downregulation encoded in the proximal promoter region, -106/+35. Progressive deletion mutants of the SSeCKS α promoter fused to luciferase reporter cassettes (left) were transiently expressed along with pRL-TK-renilla in either P69 or DU145 cells, and the resulting luciferase activity was normalized to that of renilla activity.

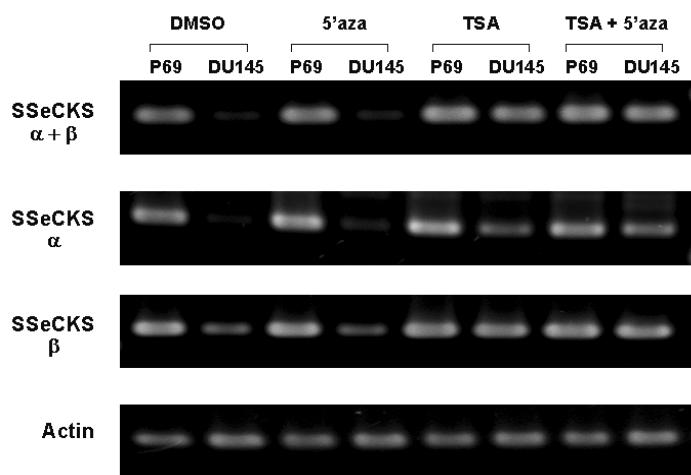


Figure 3. Semi quantitative RT-PCR of SSeCKS (either the combined α/β, or α or β transcripts, versus actin as a control) showing that TSA, but not 5-azaC, derepresses SSeCKS expression in DU145 cells.

with Src inhibitors (PP2; not shown) and SKI-606 (Fig. 7) derepresses endogenous SSeCKS message levels in DU145. Interestingly, Fig. 7 shows that the JNK inhibitor also derepressed endogenous SSeCKS expression whereas inhibitors of MEK (U1026, PD98059) or PI3K (wortmannin, LY294002), or differentiating agents (all-trans-retinoic acid, vitamin D3; not shown) had

no effect. Most of these compounds, in contrast, inhibited proliferation or oncogenic signaling of NIH3T3/v-Src cells. Moreover, pre-treatment for 3-5 days with TSA, but not with 5-aza-C, potentiated the effect of the JNK inhibitor (not shown). Thus, we are continuing to characterize these cells as candidates for the HTS. We believe we will be ready for HTS with these cells in the next several months (Task 2).

In response to Task 3 of the Statement of Work, we analyzed the cis- and trans-acting factors that control SSeCKS promoter expression in CaP cells. As a comparison, we performed a parallel analysis of the factors that control transcriptional suppression of SSeCKS in v-Src-transformed fibroblasts, with the reasoning that common, if not overlapping control mechanisms and factors would be involved. The background data are that SSeCKS steady-state transcript levels are downregulated roughly 15-fold in DU145 compared to p69 cells, or in NIH3T3/v-Src cells compared to NIH3T3. First, we identified the precise transcriptional start sites of both the human α and β promoters in P69 cells by primer-extension and RNase-protection assays (see Fig. 4 for α promoter). The transient transfection of the SSeCKS α /luciferase construct showed roughly 6- to 8-fold downregulation levels in the v-Src cells (not shown) and a 3- to 4-fold

downregulation in DU145 cells (Fig. 2) versus untransformed controls. Thus, we strongly believe that this reflects the true extent of suppression of transcriptional initiation in the transformed cells [ex.: 6-8 fold decrease in promoter activity times in Src cells 2-fold effects on stability (Fig. 5) equals roughly 15-fold total downregulation].

Using this transient expression system, we "bashed" the promoter, ultimately identifying the minimal promoter fragment of -106 to

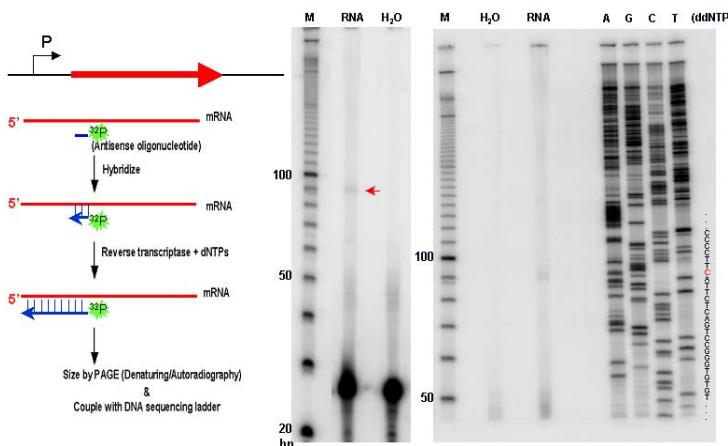


Figure 4. RNAse protection assay (compared to Sanger sequencing ladder) identifying the exact start site of the SSeCKS α promoter.

+35 as encoding the CaP- and Src-responsive sequences. Using EMSA (Fig. 6) and ChIP (not shown) assays, we showed that this promoter fragment encodes an upstream E-box that binds USF1 and a downstream GC-box that binds both Sp1 and Sp3. Interestingly, even though both boxes are required for the CaP-associated SSeCKS downregulation, binding to the downstream box is increased roughly 4-fold in the transformed cells relative to the untransformed cell nuclear lysates. At present, we are working on the hypotheses that SSeCKS downregulation is caused by either I) a relative increase in the ratio of Sp3 to Sp1 in the transformed cells- based on the notion that Sp3 encodes repressive, rather than activation functions, ii) cancer-specific post-

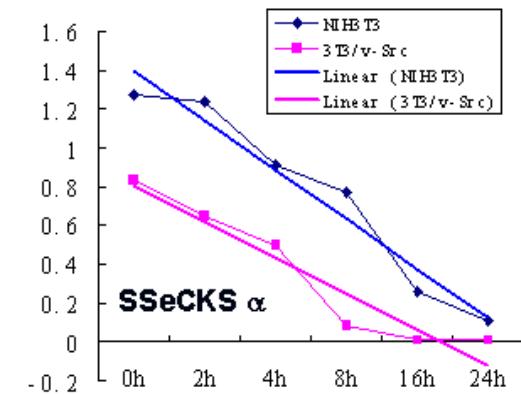


Figure 5. SSeCKS transcript stability determined by incubating cells with actinomycin D followed by RT-PCR analysis as in Fig. 3. The rate of RNA stability is defined by the 1/slope of the linear value.

translational modification of the Sp1/Sp3 complex, or iii) association of a cancer-specific repressor factor with the Sp1/Sp3 complex. Lastly, we showed by sequence comparison that the orthologous SSeCKS α and β promoters in humans, chimp, dog, cow, rat, mouse and chicken retain the same proximal promoter spacing of the E- and GC-boxes (Fig. 8), strongly suggesting that these are conserved regulatory domains throughout vertebrate evolution.

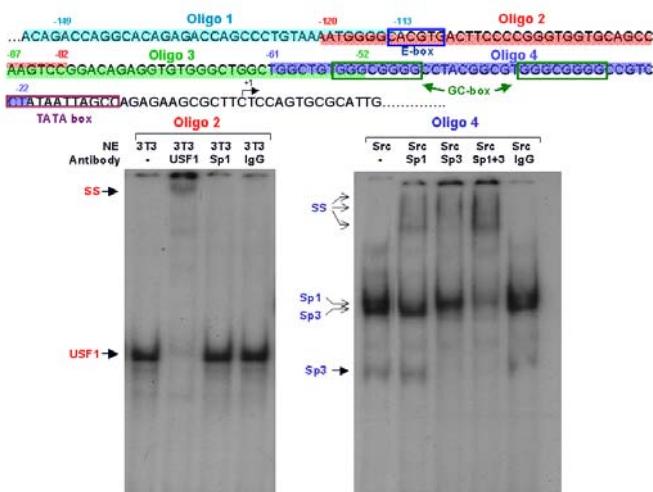


Figure 6. EMSA analysis using various oligonucleotide probes (1-4; above) of the proximal SSeCKS α promoter. Antibody supershifts showed that the E-box in oligo 2 contained USF whereas the GC-box in oligo 4 contained both Sp1 or Sp3.

Key Research Accomplishments

- construction of SSeCKS α -GFP and TK-RFP reporter plasmids
- production of indicator CaP and P69 cell lines containing the SSeCKS and TK reporter plasmids.
- development of PCR and Ab-based reagents to detect SSeCKS mRNA and protein expression changes.
- demonstration that SSeCKS/gravin/AKAP12 derepression in CaP cells can be induced by TSA but not by 5-aza-C.
- demonstration that of the roughly 15-fold decrease in SSeCKS transcript levels in CaP vs. normal cells, 2-fold is controlled by decreases in transcript stability whereas the remaining portion is controlled by a 6- to 8-fold decrease in promoter activity levels.
- demonstration that the minimal CaP- and Src-responsive portion of the SSeCKS promoter is encoded between -106 and +35.
- identification of requirements for both upstream E- and downstream GC-box motifs for downregulation.
- demonstration that the E-box is occupied by USF1 (and not, for example, Myc) and that the GC-box is occupied by a combination of Sp1 and Sp3 (and not, MAZ).

In sum, we have made major strides in Task 3 and even have begun earlier than contemplated to start the experiments in Task 4, namely, to identify the transcription factors involved in CaP-associated SSeCKS downregulation.

Based on our preliminary data showing that methylation does not play a major role in CaP-associated SSeCKS downregulation, but that histone deacetylation does, we have redirected much of our aims in Task 5 in order to characterize the deacetylases (HDAC) involved as well as the transacting factors that recruit the HDACs to the SSeCKS promoter region.

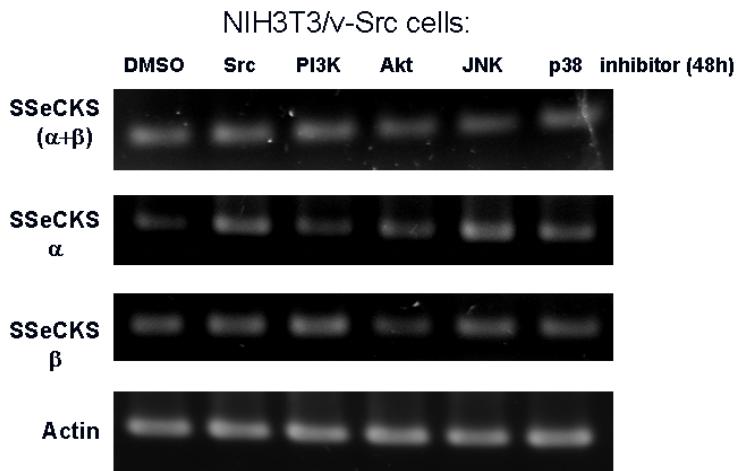


Figure 7. Src or JNK inhibitors derepress expression of SSeCKS α or β transcripts.

-demonstration that the level of USF1 does not vary in CaP vs. normal cells, but that there is a relative 4-fold increase in Sp3:Sp1 in the transformed cells.

Reportable Outcomes

-Poster report, 2005 Oncogene Meeting, Frederick, MD, "Mapping of v-Src- and prostate cancer-responsive control sequences to the SSeCKS proximal promoter", Bu, Y. and Gelman, I.H., 6/21-24/2005.

-Poster report, 2006 Annual Meeting, American Assoc. of Cancer Research, Washington, DC, "Identification of v-Src- and prostate cancer-responsive sequences in the promoters of SSeCKS/Gravin/AKAP12, a metastasis-suppressor gene", Bu, Y. and Gelman, I.H., 4/1-5/2006.

-Development of CaP indicator cell lines and probes for SSeCKS isoform expression.

Conclusions

We have made major inroads in producing and characterizing the indicator lines we will use for the HTS drug screening, in characterizing the cis- and trans-factors controlling SSeCKS downregulation in CaP cells, and in elucidating the molecular mechanisms and pathways involved in SSeCKS transcriptional control.

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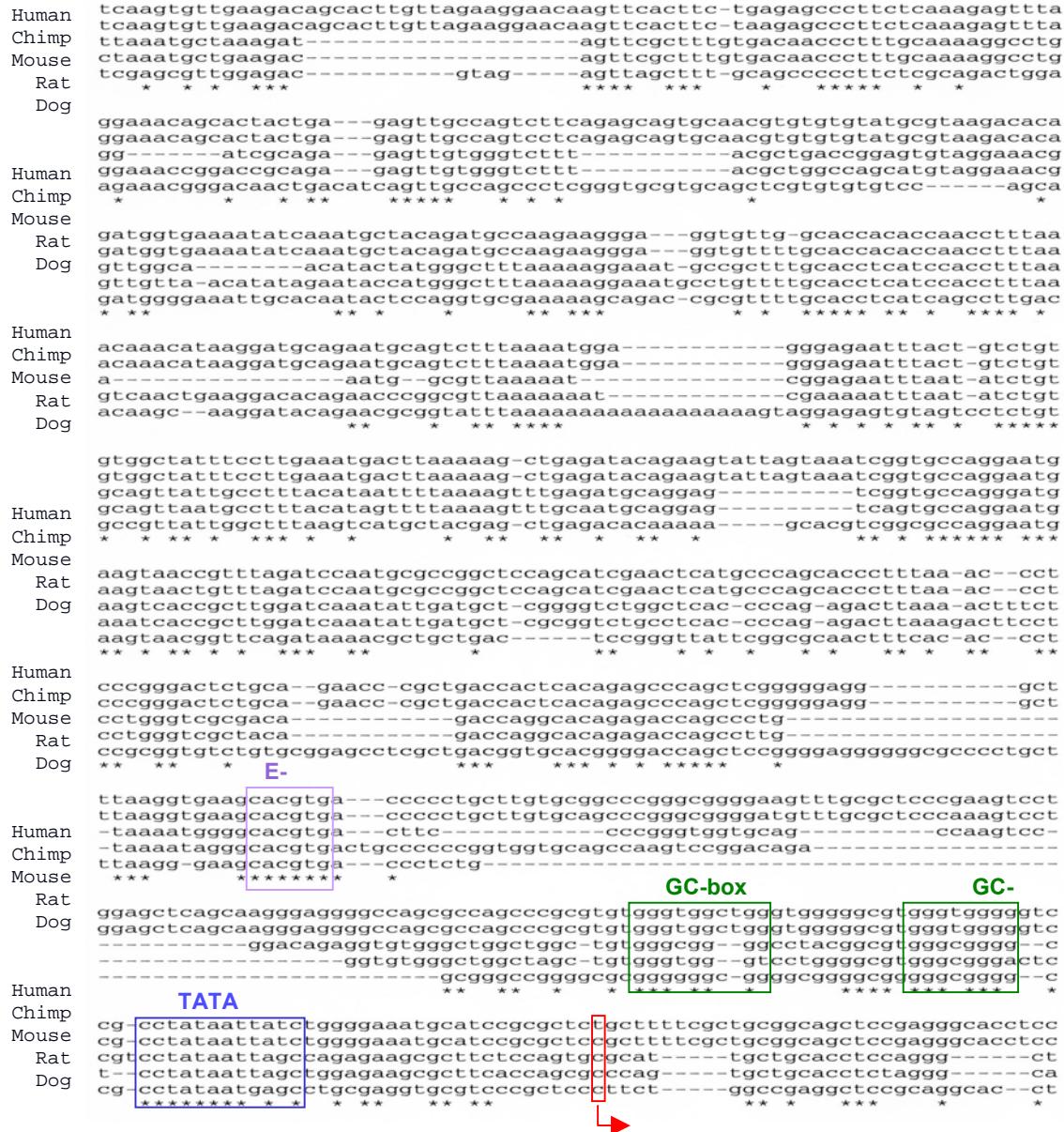


Figure 8. Sequence alignment of SSeCKS α promoter regions in various mammalian species showing strong sequence conservation (*), especially in the retention and spacing of the E- and GC-box motifs just proximal to the transcriptional start site (red).